European Journal of Pharmaceutics and Biopharmaceutics 83 (2013) 396–404

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

An antioxidant regenerating system for continuous quenching of free radicals in chronic wounds

Gibson S. Nyanhongo [a,*], Christoph Sygmund [b], Roland Ludwig [b], Endry Nugroho Prasetyo [a], Georg M. Guebitz [a]

[a] Graz University of Technology, Institute of Environmental Biotechnology, Graz, Austria
[b] BOKU – University of Natural Resources and Life Sciences, Department of Food Sciences and Technology, Vienna, Austria

ARTICLE INFO

Article history:
Received 3 July 2012
Accepted in revised form 7 October 2012
Available online 12 November 2012

Keywords:
Free radicals
Free radical generating system
Antioxidants
Antioxidant regenerating system
Cellobiose dehydrogenase
Cellobiose

ABSTRACT

A novel antioxidant regenerating system consisting of cellobiose dehydrogenase (CDH), cellobiose, and phenolic antioxidants with potential application for continuous quenching of free radical species in chronic wounds was developed. This antioxidant regenerating system, continuously quenched *in situ* produced ·NO, $O_2^{\cdot-}$ and OH· radicals and the produced oxidized phenolic antioxidants were regenerated back to their original parent compounds by CDH using cellobiose as electron donor. This system therefore prevented the accumulation of oxidized phenolic antioxidants. Interestingly, this study also challenges the relevance of using total antioxidant capacities values of plant crude extracts obtained using biologically none relevant radical species like (2,2-diphenyl-1-picrylhydrazyl (DPPH)), Trolox Equivalent Antioxidant Capacity (TEAC), etc. when applied as medicinal remedies. This is because methoxylated phenolic antioxidants like sinapic acid, ferulic acid; 2,6-dimethoxyphenol readily donate their electrons to these radicals (DPPH, TEAC, etc.), thereby greatly influencing the total antioxidant values although this study showed that they are not at all effective in quenching $O_2^{\cdot-}$ radicals and again are not the most effective quenchers of ·NO and OH· radicals as demonstrated during this study.

1. Introduction

Molecular oxygen plays a crucial role in sustaining life on this planet through its involvement in many processes, including photosynthesis and aerobic respiration. Nevertheless, O_2 which exists as a biradical molecule is also toxic especially when converted into superoxide ($O_2^{\cdot-}$) radicals which are considered the primary reactive oxygen species (ROS) in biological systems. In aqueous solution, $O_2^{\cdot-}$ spontaneously dismutates to hydrogen peroxide (H_2O_2), while in the presence of metals, for example, copper and iron, H_2O_2 and $O_2^{\cdot-}$ generate hydroxyl radicals (OH·) radicals [1,2]. $O_2^{\cdot-}$ also react with nitric oxide (·NO) to yield reactive nitrogen oxygen species (RNS) for example, peroxynitrite ($ONOO^-$), a powerful oxidant that oxidizes biomolecules. Although these free radicals (ROS and RNS) are byproducts of normal aerobic metabolism, and their production is relatively increased during infection [1,3], persistent overproduction is the major factor driving wounds into chronic state. This is because these free radicals continuously oxidize newly synthesized biomolecules in chronic wounds and also act as promoters of pro-inflammatory responses. Therefore, chronic wounds defined as wounds that do not heal, remain in the inflammatory phase for an unpredictable time [4–6]. Chronic wounds constitute a major health care challenge all over the world, with the global economic burden estimated to be between US $13 and $15 billion annually [5–7].

Although reasonable progress has been achieved in controlling wound exudates, protecting wound from further physical damage, infection and preventing loss of blood [8–14], the control of inflammation, proteases, and free radicals to promote wound healing remain a big challenge. In our opinion, success in chronic wound healing will only be achieved when the physiological balance similar to that in acute wounds is restored. This includes measures like incorporating systems which continuously scavenge/quench ROS and RNS into the bioactive wound dressing polymers. Given the unquestionable importance of ·NO in promoting wound healing (participation in angiogenesis, inflammation, cell proliferation, matrix deposition and remodeling) [15–17], as part of the strategy to promote chronic wound healing, its level should be reduced to optimal concentrations, while its reaction with $O_2^{\cdot-}$ during the "respiratory burst" should be suppressed. This can be achieved by incorporating carefully selected plant phenolics antioxidants, which react selectively with specific free radicals protecting biological systems from oxidative damage. Plant phenolics are

* Corresponding author. Graz University of Technology, Institute of Environmental Biotechnology, Petersgasse 12/1, A-8010 Graz, Austria. Tel.: +43 873 4313; fax: +43 316873 8819.
E-mail addresses: g.nyanhongo@tugraz.at, gnyanhongo@yahoo.com (G.S. Nyanhongo).

0939-6411/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejpb.2012.10.013

increasingly being used as medicinal remedies for many pathological conditions, including as antimicrobial and antioxidant compounds [18–26]. However, once phenolics antioxidants quench ROS and RNS, they themselves become reactive species too (phenoxy radicals, radical quinones or quinones), a condition that drive the wound environment toward oxidative stress with time and also promote oxidation of biomolecules [27]. This also prevents successful healing of chronic wounds.

To prevent the accumulation of the free radicals (ROS and RNS), a novel system which relies on cellobiose dehydrogenase (CDH), a versatile enzyme able to reduce phenoxy radicals, radical quinones or quinones [28–31], and plant phenolic antioxidants is reported for the first time. The system is designed in such a way that the phenolic antioxidant molecules quench ROS and RNOS ($O_2^{\cdot-}$, OH·, ·NO, etc.), and the oxidized phenolic antioxidants are then reduced back to their original parent compound by CDH using cellobiose as electron donor (Fig. 1). It is our strong belief that such a system when incorporated into wound dressing hydrogels will guarantee a continuous supply of antioxidants. These antioxidants will continuously quench the free radicals, prevent the oxidation of the newly synthesized biomolecules, suppress the persistent expression of pro-inflammatory responses, and also prevent the accumulation of oxidized phenolics. This system combined with many other available medication regimes aimed at modulating pro-inflammatory gene expression system, removal of exudates, inhibiting proteases, inhibiting pathogenic microorganisms will help create ideal conditions for the successful healing of chronic wounds.

CDH is the only currently known extracellular flavocytochrome, which uses a variety of oligosaccharides substrates as electron donors (cellobiose, lactose, triose, and many cellulose fragments) to reduce many structurally different phenoxy radicals, radical quinones or quinones (electron acceptors) [28–31]. The versatile electron transfer properties of CDH are being exploited for many biotechnological applications including the development of biosensors for the detection of cellodextrins [32] maltose [33], lactose [34], diphenolic compounds [35–37], and catecholamines (dopamine, adrenaline and noradrenaline) [38]. CDH has been shown to continuously regenerate laccase oxidized substrates [39]. It is therefore believed that this system could play a crucial role in recycling phenolic antioxidants in chronic wounds through their strategic incorporation into wound dressing polymers like hydrogels.

2. Materials and methods

2.1. Materials

Potassium persulfate (PMS), ethylenediamine tetraacetic acid (EDTA), ascorbic acid, 2-deoxy-2-ribose, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), 2,6-dichlorophenol-indophenol (DCIP), phenazine methosulfate (PMS), sodium nitroprusside (SNP), H_2O_2, ascorbic acid, iron sulfanilamide, naphthylethylenediamine dihydrochloride (NED), H_2O_2, NASH reagents (2,4-pentanedione, acetic acid, ammonium acetate), including phenolic antioxidants (2,6-dimethoxyphenol; guaiacol, catechol, sinapic acid, gallic acid, ferulic acid, caffeic acid, vanillic acid) were purchased from Sigma–Aldrich. All the other chemicals were purchased from Merck. *Myriococcum thermophilum* cellobiose dehydrogenase was produced and purified as previously described Flitsch et al. [40].

2.2. Cellobiose dehydrogenase (CDH) activity assay

The activity of CDH was assayed according to Baminger et al. [41] with slight modifications. Briefly, CDH activity was measured by monitoring the decrease in absorbance of 2,6-dichlorophenol-indophenol (DCIP), at 520 nm ($\varepsilon_{520} = 6.8 \times 10^3\ M^{-1}\ cm^{-1}$), pH 4.0

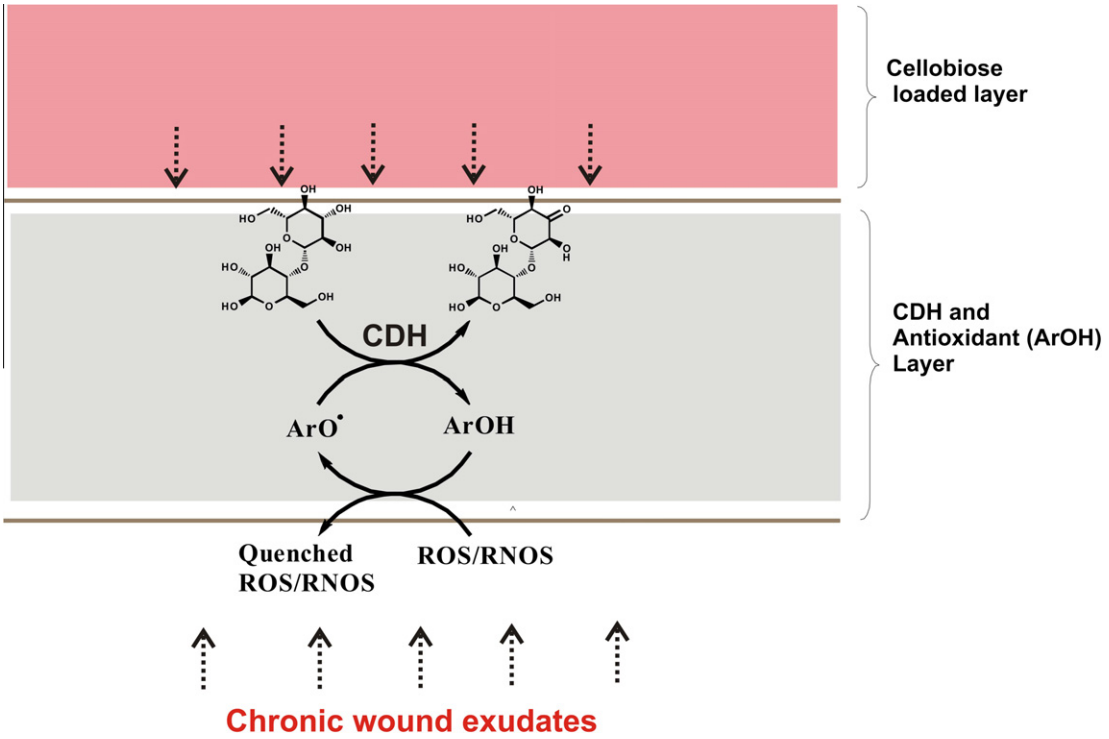

Fig. 1. Schematic representation of the antioxidant regenerating system. The phenolic antioxidants quench ROS and RNOS. The resulting oxidized phenolic antioxidants are then reduced back to their original compound by CDH using cellobiose as an electron acceptor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and 37 °C using a Hitachi UV–VIS 2001 spectroscopy. The reaction mixture (in a total volume of 1 ml) contained the following: 0.3 mM DCIP, 30 mM cellobiose 100 mM sodium acetate buffer, pH 4.0). After temperature adjustment, the reaction was started by addition of CDH enzyme of final activity 5 U ml^{-1}, and the decrease in absorbance was monitored during the first 2 min. One unit of enzyme activity is defined as the amount of enzyme reducing 1 μmol of DCIP per second under the above reaction conditions.

2.3. Regeneration of oxidized antioxidants by CDH

The ·NO radicals were used as a model to demonstrate the ability of CDH to quench oxidized phenolic antioxidants. At physiological pH, ·NO is generated from aqueous sodium nitroprusside (SNP) solution and interacts with oxygen to produce nitrite ions, which were quantified using the Griess reagent [42]. Quenching of the generated ·NO was tested with various antioxidants in presence or absence of CDH to regenerate the antioxidants. The reaction mixture (1 ml) contained 1 mM SNP in PBS buffer (pH 7.4) with 100 μM of antioxidant (either 2,6-dimethoxyphenol; guaiacol, catechol, sinapic acid, gallic acid, ferulic acid, caffeic acid or vanillic acid) with or without antioxidant regenerating system (5 U ml^{-1} CDH + 400 μM cellobiose) final concentration. The reaction mixture was then incubated at 25 °C for 150 min in front of a visible 25 W tungsten lamp. After incubation for 150 min at 25 °C, 0.5 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Further, 0.5 ml of naphthylethylenediamine dihydrochloride (0.1% w/v) was also added, and the mixture incubated for 30 min at 25 °C. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured at 540 nm against a blank sample [43]. The generated nitrite was estimated using a standard curve based on known concentrations of sodium nitrite solutions.

3. HPLC–MS analysis of the ability of CDH to regenerate oxidized phenolic antioxidants

In addition to UV–VIS monitoring of antioxidant regenerating system, HPLC was carried out using Dionex HPLC-UVD-system equipped with a P580 pump, an ASI-100 autosampler, and a PDA-100 photodiode array detector. Reaction mixtures containing phenolic antioxidants NO generating system prepared as described above were incubated simultaneously with or without an antioxidant regenerating system. An equal volume of methanol was added, the mixture was centrifuged for 15 min at 14,000*g*, and 600 μl aliquots were transferred into HPLC vials. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (XBridge, C18, 150 × 4.6 mm, Water, USA) using a linear gradient of methanol (solvent B) and 0.1% Formic acid (solvent A) as solvent at a flow rate of 1 ml min^{1}, an injection volume of 10 μl, and an oven temperature of 30 °C.

3.1. Quenching of superoxide ($O_2^{\cdot-}$) by the antioxidant regenerating system

The ability of CDH/antioxidant system to quench $O_2^{\cdot-}$ radicals was investigated by monitoring the inhibition of the reduction of nitro blue tetrazolium (NBT). The phenazine methosulfate – nicotinamide adenine dinucleotide (PMS/NADH) system generates $O_2^{\cdot-}$ radicals which reduce nitro blue tetrazolium (NBT) to a purple formazan product monitored at 560 nm. However, the presence of an effective $O_2^{\cdot-}$ radicals quencher prevents the reduction of NBT. The reaction mixture contained phosphate buffer 100 μM NADH, 120 μM NBT, 100 μM PMS supplemented with either 50 μM of the antioxidant (either 2,6-dimethoxyphenol; guaiacol, catechol, sinapic acid, gallic acid, ferulic acid, caffeic acid or vanillic acid) alone or antioxidant regenerating system (50 μM phenolic antioxidant, 100 μM cellobiose and CDH of 5 U final activity).

3.2. Quenching of OH· radicals by the antioxidant regenerating system

The ability of the CDH/antioxidant regenerating system to quench OH· radicals was investigated by incubating a OH· generating system (H_2O_2–ascorbic acid–iron) with 100 μM antioxidants (2,6-dimethoxyphenol; guaiacol, catechol, sinapic acid, gallic acid, ferulic acid, caffeic acid, vanillic acid) or respective phenolic antioxidant regenerating system. The CDH–phenolic antioxidant regenerating system consisted of 200 μM cellobiose and CDH of 5 U final activity. The reaction mixture, in a total of volume 3 ml, contained 100 mM phosphate buffer (pH 7.4), radical generating system (200 μM H_2O_2, 200 μM iron −1 mM ascorbic acid complex) and 40 mM DMSO final concentration with or without the antioxidants. Mannitol, a classical OH· scavenger, was used as a positive control. The reaction mixtures were incubated at 37 °C for 1 h. After 1 h incubation, the inhibition of the formation of formaldehyde due to the decreased formation of OH· was assayed spectrophotometrically using the Nash method [44]. Percentage inhibition was evaluated by comparing the test and blank solutions.

3.3. Data analysis

Unless otherwise specified, quenching of $O_2^{\cdot-}$, OH· and ·NO was estimated by comparing the concentration of oxidized indicators molecules in the presence of the radical generating system alone with those of the reaction mixture containing antioxidant or both antioxidant and antioxidant regenerating system. The results were then expressed as percent quenching as shown in following equations:

$$\% \text{ Quenched free radicals} = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

$$\% \text{ Quenched free radicals} = \frac{A_1 - A_2}{A_1} \times 100 \quad (2)$$

where A_0 is the concentration of oxidized or reduced substrates by the radical generating system, and A_1 is the concentration of oxidized or reduced substrates in the presence of alone, and A_2 is the concentration of oxidized or reduced substrates in the presence of both antioxidant and antioxidant regenerating system.

4. Results and discussions

4.1. ROS and RNS quenching with the CDH–cellobiose–phenolic antioxidant regenerating system

Free radicals (·NO, $O_2^{\cdot-}$ and OH· radicals) generating systems were incubated with several well-known phenolic antioxidants including catechol, caffeic acid, sinapic acid, gallic acid, guaiacol, ferulic acid, and vanillic acid in the presence or absence of an antioxidant regenerating system (cellobiose plus the CDH enzyme). As exemplified by nitric oxide ·NO), all tested antioxidants quenched the radicals resulting in their oxidation (Fig. 2). Upon addition of CDH and cellobiose, the oxidized antioxidants were reduced resulting in their regeneration (Fig. 2).

Further proof of the ability of CDH to regenerate the oxidized phenolic antioxidants was provided by HPLC analysis as evidenced for vanillic acid and 2,6-dimethoxyphenol (Fig. 3). There was

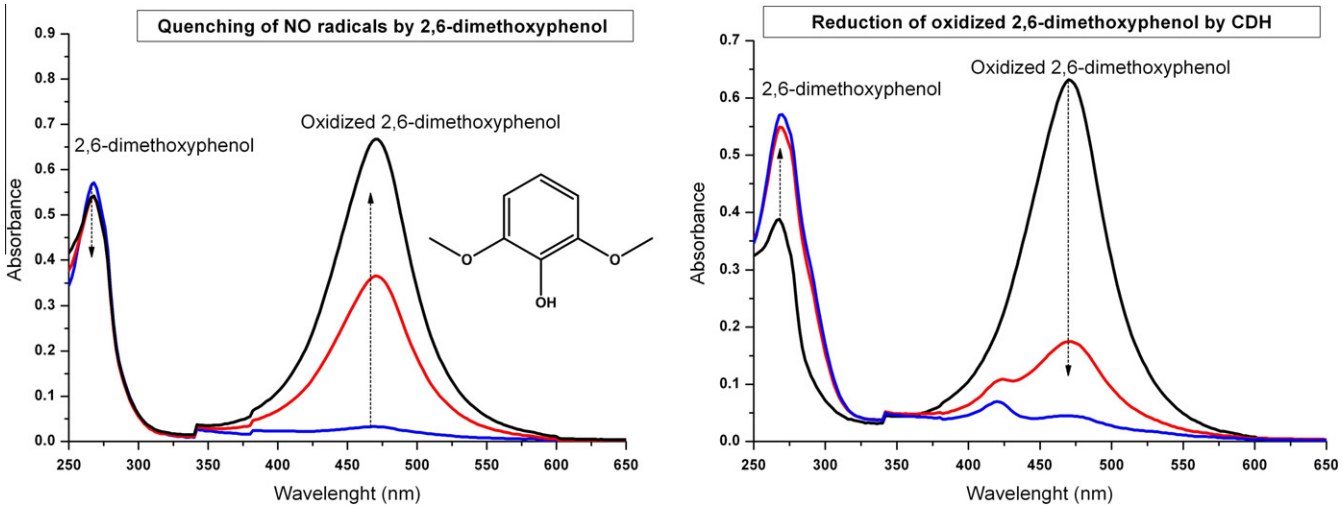

Fig. 2. Wavelength scan profiles of the quenching of NO radicals by different phenolic antioxidants and the subsequent regeneration of oxidized antioxidants by CDH using cellobiose as electron donor. NO generating system was incubated with antioxidants for 45 min, and then the antioxidant regenerating system was added and further incubated for 20 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

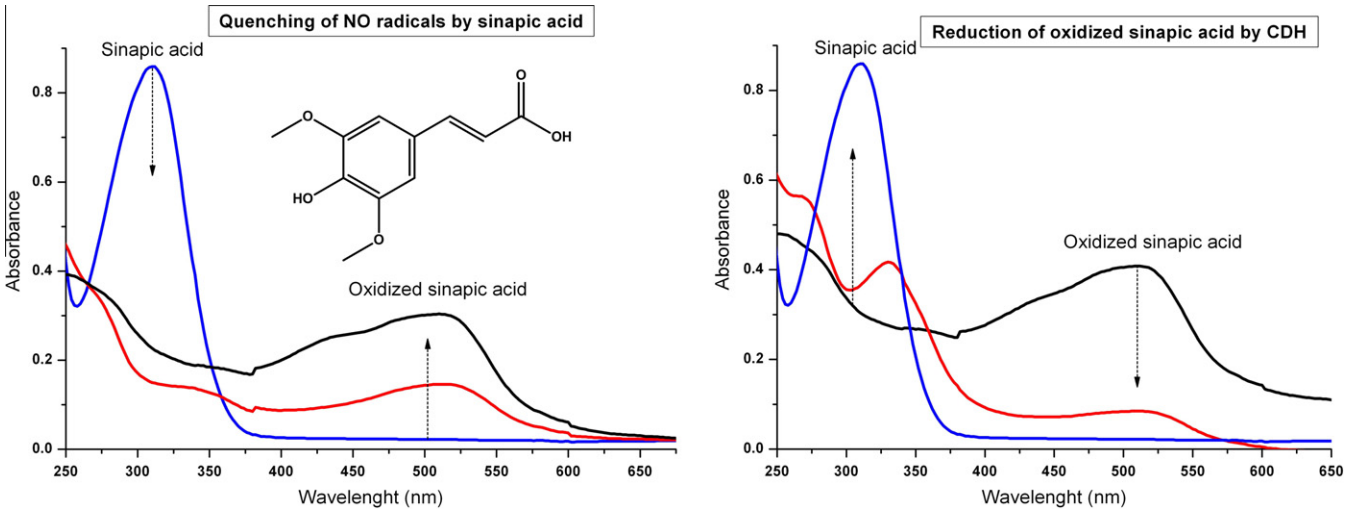

Fig. 3. HPLC-analysis of oxidation/reduction products of phenolic antioxidants during incubation with NO radical generating system. Quenching of NO radicals by phenolic antioxidants (DMP and catechol) or ascorbic acid and their regeneration by CDH in the presence of cellobiose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complete disappearance of vanillic acid and reduction in 2,6-dimethoxyphenol incubated with the radical generating system without the antioxidant regenerating system or ascorbic acid. However, the reduction in these antioxidants was insignificant in absence of the antioxidant regenerating system (Fig. 3). This is a clear demonstration that CDH was able to use cellobiose as electron donor to reduce oxidized phenolic antioxidants. Similarly, addition of ascorbic acid to oxidized antioxidants resulted in their reduction (Fig. 3). However, it should also be noted that some antioxidants could be lost due to the polymerization or formation of new products of oxidized phenolic antioxidants, and this is also clearly demonstrated by the small peaks (appearing between 14.5 and 14.60 in the HPLC chromatograms of 2,6-dimethoxyphenol incubated with the radical generating system alone (Fig. 3). Once oxidized phenolic compounds are well-known to form intramolecular or intermolecular coupling products [45,46]. Therefore, to prevent this coupling, it is important to design the system such that once the antioxidant quenches a radical, it is immediately reduced by the antioxidant regenerating. This can be achieved by supplying the antioxidant regenerating system in excess.

Interestingly, during incubation of vanillic acid with ·NO, two peaks, one absorbing around 400 nm and the other at 700 nm appeared in the chromatogram (Fig. 2). Surprisingly, upon addition of the antioxidant regenerating system, only the peak appearing around 400 nm disappeared while that at 700 nm remained constant (Fig. 2). Addition of ascorbic acid or trolox, well-known antioxidants did not also affect this peak at 700 nm. This peak (700 nm) may be attributed to the reaction of ·NO with vanillic acid. This is supported by previous studies, which demonstrated that ·NO coupled phenolic compounds lost their antioxidant properties [47,48]. Although some authors have reported nitrosation as a negligible phenomenon [49], screening against such molecules is important since the phenolic amines generated are well-known carcinogens. Thus, molecules like vanillic acid should be avoided for incorporation in the bioactive chronic wound dressing polymers.

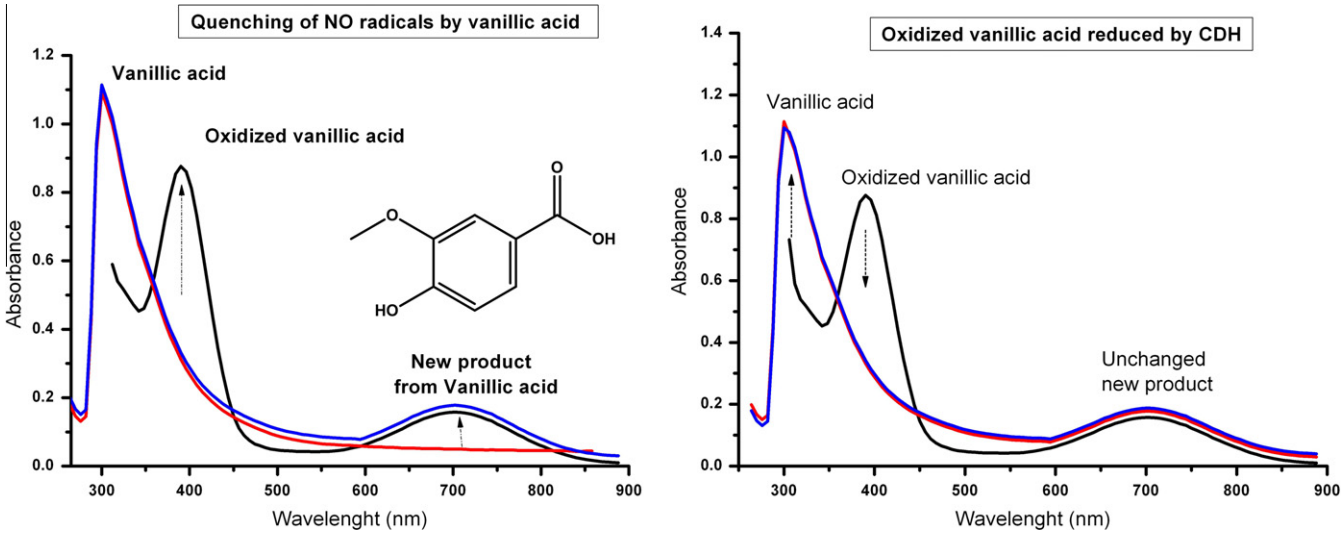

Fig. 4. Quenching of NO radicals with phenolic antioxidants in presence of absence of CDH and cellobiose. Quenching is expressed as% inhibition of the formation of 925 μM nitrite ions in samples incubated with a NO generating system. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Comparing the ·NO quenching abilities of the phenolic antioxidant system alone and the CDH–antioxidant regenerating system shows that the later was consistently more effective in inhibiting the formation of 925 μM nitrite ions regardless of the phenolic antioxidant (Fig. 4). However, the overall effectiveness depended on the ability of the individual antioxidant to quench ·NO. For example, hydroxylated phenolic antioxidants catechol, gallic acid, and ferulic acid were among the best in the presence and absence of the antioxidant regenerating system (Fig. 4).

Their ability to quench ·NO increased above 70% in the presence of the antioxidant regenerating system (Fig. 4). Previous studies have also reported catechol [50,51] and caffeic acid [52] as effective ·NO quenchers. Methoxylated phenolic antioxidants like 2,6-dimethylphenol as also shown in this study were also reported to be weak ·NO scavengers [47,48]. The ability of CDH to reduce guaiacol and vanillic acid radicals was already reported by Ander and coworkers [53] as early as 1993 although this was never exploited for regenerating antioxidants. It is worth noting that the presence of the antioxidant regenerating system led to the quenching of higher amounts of ·NO (Fig. 4).

4.2. Quenching of $O_2^{\cdot-}$ by the CDH–cellobiose–phenolic antioxidant regenerating system

The PMS/NADH system was used to generate $O_2^{\cdot-}$ *in vitro* in the presence of phenolic antioxidant alone or the CDH-based antioxidant regenerating antioxidant system supplemented with NBT. The effectiveness of the antioxidant or antioxidant regenerating antioxidant system was measured as its ability to prevent the reduction of NBT to yield formazan by the *in situ* generated $O_2^{\cdot-}$. As shown in Fig. 5, the 120 μM NBT incubated with the $O_2^{\cdot-}$ radical generating systems were reduced, while 32, 57, and 76 μM NBT was reduced upon incubation with $O_2^{\cdot-}$ radical generating system in the presence of gallic acid, catechol and caffeic acid, respectively. The $O_2^{\cdot-}$ quenching ability therefore increased with increasing hydroxyl groups when comparing gallic acid and catechol. However, sinapic acid, ferulic acid, and 2,6-dimethoxyphenol, well-known antioxidants using total antioxidant capacity assay methods like DPPH, ABTS, *N,N*-dimethyl-*p*-phenylenediamine (DMPD), etc., were not able to quench $O_2^{\cdot-}$ radicals.

Comparing these methoxylated phenolics shows that the increasing number of methoxyl groups decreases $O_2^{\cdot-}$ radicals quenching activity. Aboul-Enein et al. [54] also reported weak $O_2^{\cdot-}$ scavenging activity of ferulic acid and also demonstrated that dihydroxy benzoic acids were good $O_2^{\cdot-}$ quenchers. This observation shows that the total antioxidant capacity values of plants crude extracts obtained using many of the available methods like DPPH, ABTS, TMAMQ, etc. do not reflect the ability of these crude extracts in scavenging $O_2^{\cdot-}$ radicals since the obtained values are greatly influenced by methoxylated phenolics. This shows that it is not enough to just refer to the total antioxidant capacity of plant crude extracts when this is directed at *in vivo* treatment of oxidative stress in patients. This observation also helps to partly justify the many conflicting reports on the role of plant phenolic antioxidants as remedies for oxidative stress related diseases.

Further incorporation of the antioxidant regenerating system (CDH + cellobiose) during incubation of the $O_2^{\cdot-}$ radical generating system with caffeic acid, catechol or gallic acid enhanced their radicals quenching abilities (Fig. 6). Under the various conditions investigated, the CDH–phenolic antioxidant regenerating system consistently performed better (quenched more $O_2^{\cdot-}$, thereby preventing the reduction of NBT) than the system containing the phenolic antioxidant alone (Fig. 6).

The CDH–gallic acid quenching system increased the $O_2^{\cdot-}$ quenching activity by 36% as compared to gallic acid alone. Similarly the catechol–CDH radical scavenging system, increased $O_2^{\cdot-}$ quenching activity by 43%. Further studies performed by spiking the radical quenching system during the production of $O_2^{\cdot-}$ by the (PMS/NADH) system showed similar trend (Fig. 7).

For example, spiking CDH and gallic acid, catechol or caffeic acid led to the immediate decrease in the reduction of NBT (Fig. 8). The rate of reduction of NBT was decreased from an average 1.05 μM s^{-1} in the absence of the radical quencher to 1.24×10^{-1} - μM s^{-1}, 1.62×10^{-1} μM s^{-1} and 3.32×10^{-1} μM s^{-1} for gallic acid, catechol and caffeic acid, respectively. In contrast, the CDH–phenolic antioxidant regenerating system constituted with the methoxylated phenolics (sinapic acid, vannilic acid, 2,6-dimethoxylphenol) were not at all effective in moping $O_2^{\cdot-}$ radicals as evidenced by the increased reduction of NBT similar to the control (Fig. 8). These results also show CDH and cellobiose have no effect as long as there were no oxidized phenolic antioxidants. Nevertheless, what is very

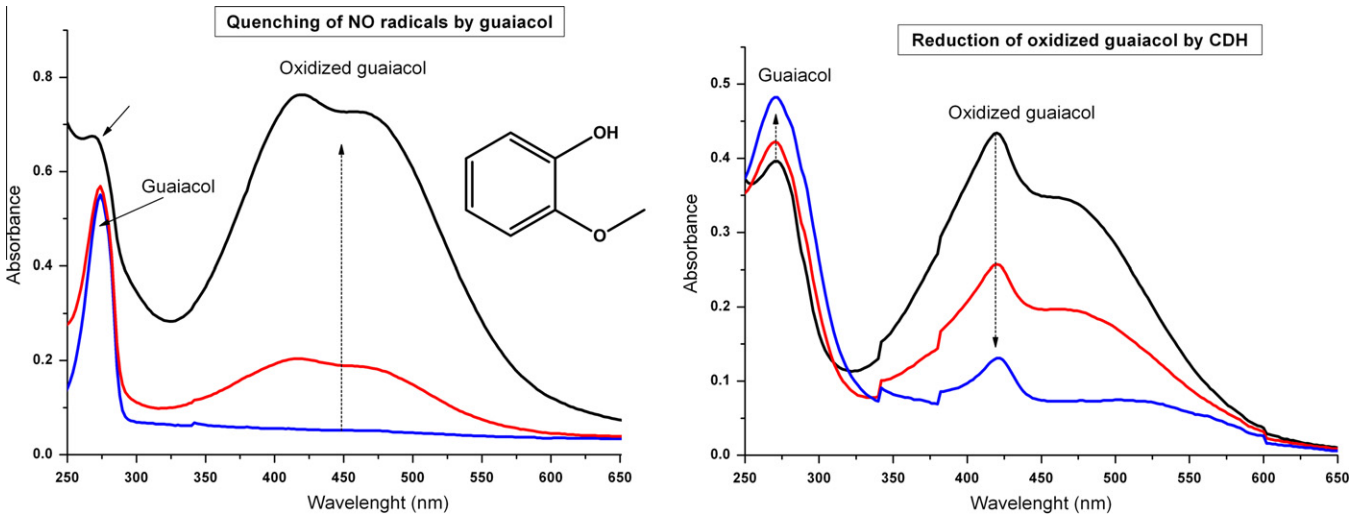

Fig. 5. Quenching of *in situ* generated $O_2^{\cdot-}$ radicals with phenolic antioxidants. A higher quenching results in a lower amounts of formazan formation since the antioxidant prevent the reduction of NBT to formazan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

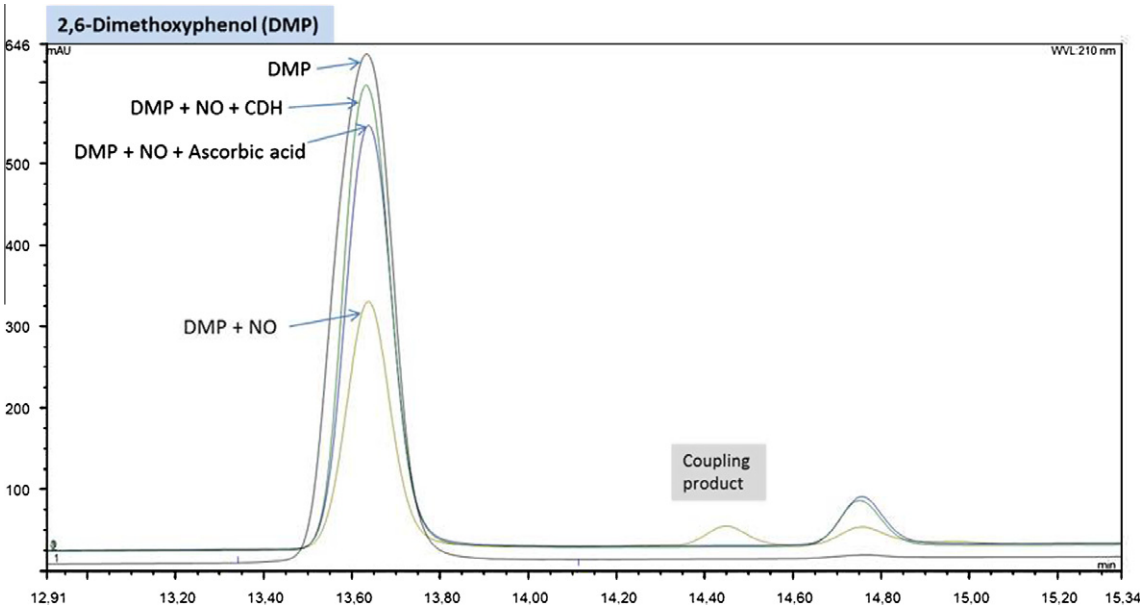

Fig. 6. Quenching of *in situ* generated $O_2^{\cdot-}$ radicals with phenolic antioxidants in presence of absence of CDH and cellobiose. Higher quenching results in lower amounts of formazan formation since the antioxidant prevent the reduction of NBT to formazan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clear from the results in Figs. 2 and 3, is that the phenolic radicals generated after quenching ·NO or $O_2^{\cdot-}$ are reduced back to their original parent compound and continues to quench the generated radicals. Judging by the results of this study, effective quenching of $O_2^{\cdot-}$ radicals in chronic wounds is preferably achieved by incorporating phenolic compounds rich in hydroxyl groups like gallic acid and catechol as well as the antioxidant regenerating system. Further, these studies show that the general agreed proposition based on observing the reaction of phenolic antioxidants with none biological radicals such as ABTS and DPPH, that is, the substitution of one or two methoxy groups at the *ortho* position relative to the hydroxyl group markedly increases the antioxidant activity of phenolic acids (e.g., sinapic acid is a more efficient antioxidant than ferulic acid) [55], is irrelevant with respect to $O_2^{\cdot-}$ radicals and does not also fully apply to ·NO radicals. For example, the presence of methoxyl groups at *meta* and *para* positions in hydroxybenzoic acids increased the antioxidant activity when comparing vannilic acid and syringic acid and a similar trend was also observed for hydroxycinnamates when comparing caffeic acid and sinapic acid [20,56], yet these methoxylated phenolics are poor $O_2^{\cdot-}$ scavengers. Similarly, the belief that hydroxylated cinnamic acids are more effective antioxidants than their benzoic acid counterparts [55] is a relative term which again does not apply in quenching $O_2^{\cdot-}$ radicals. However, it is agreed that increasing hydroxyl groups in phenolic antioxidant increases the antioxidant activity of phenolic acids (gallic acid with three hydroxyl groups is more active than catechol) toward $O_2^{\cdot-}$ and ·NO. By targeting the scavenging of $O_2^{\cdot-}$, the production of deleterious ROS and RNOS like $ONOO^-$ or OH· will be curtailed. ·NO is the only known biological molecule that outcompetes endogenous superoxide dismutase in reacting with $O_2^{\cdot-}$ [17], and under inflammatory conditions, simultaneous production of $O_2^{\cdot-}$ and ·NO has been shown to increase the formation

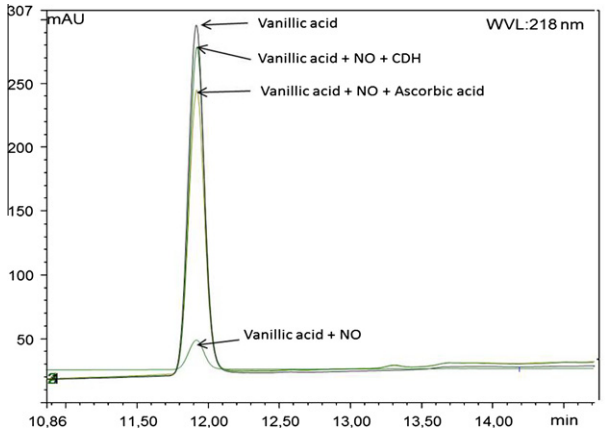

Fig. 7. The $O_2^{\cdot-}$ generating (PMS/NADH) system was incubated with antioxidant molecules alone or the CDH-phenolic antioxidant regenerating system and the % inhibition of the reduction of 120 μM NBT measured and compared with the control ($O_2^{\cdot-}$ generating system and 120 μM NBT). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

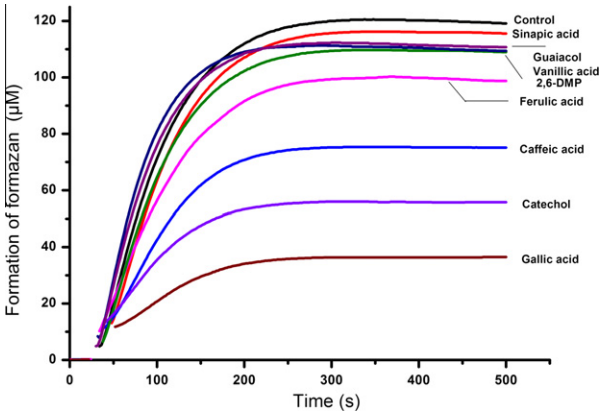

Fig. 9. Quenching of *in situ* generated OH· radicals with phenolic antioxidants in presence of absence of CDH and cellobiose. A higher quenching results in a lower oxidation of 40 mM DMSO by the OH· generating system (H_2O_2–ascorbic acid–iron–EDTA) resulting in a lower amount of formaldehyde. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

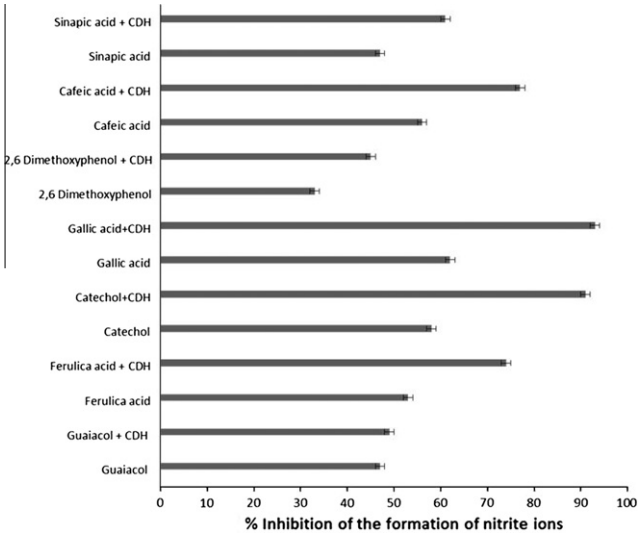

Fig. 8. Effect of spiking CDH-phenolic antioxidants regenerating system on the rate of reduction of NBT to formazan during *in situ* incubation of NBT with $O_2^{\cdot-}$ radicals. CDH-phenolic antioxidants regenerating system was spiked during the exponential reduction of NBT.

of $ONOO^-$ by a 1,000,000-fold [17]. This therefore emphasizes the need to incorporate an effective $O_2^{\cdot-}$ quenching system to prevent the formation of $ONOO^-$ and its decomposition products which continually oxidize biomolecules in the chronic wounds [57].

4.3. Quenching of OH· radicals by the CDH-cellobiose–phenolic antioxidant regenerating system

The OH· radicals is an extremely reactive free radical which reacts rapidly with almost evertype of molecule found in living cells including sugars, proteins, lipids, and DNA. Therefore, strategies aimed at preventing its formation by for example providing an effective $O_2^{\cdot-}$ radicals or its immediate removal will be of great benefit. As shown in Fig. 9, gallic acid (95% inhibition) and catechol (91% inhibition) were the most effective in quenching OH· radicals as compared to methoxylated phenolics (Fig. 9). The OH· radicals quenching ability of CDH–phenolic antioxidant regenerating was estimated based on preventing the conversion of DMSO to formaldehyde. The methoxylated phenolic sinapic acid, guaiacol, and 2,6-dimethoxyphenol were not effective in the preventing the oxidation of DMSO. Interestingly, the ability to quench OH· radicals, thereby inhibiting the production of formaldehyde was significantly enhanced by incorporating the antioxidant regenerating system (CDH + cellobiose) in all the experimental setups. The efficiency followed the same trend as observed in the presence of the antioxidant alone (Fig. 9). Comparing gallic acid and catechol, it would also appear that the ability to quench OH· radicals slightly increased with increasing number of hydroxyl groups (Fig. 9). Cheng et al. [58] also noted that mono-hydroxylated phenolic compounds were less active than those having two hydroxyl groups on the benzene ring. Further, it has also been confirmed that when phenolic antioxidants are *ortho* and *para* substituted as in catechol and caffeic acid their OH· radicals activity is even increased further as reported by Thavasi et al. [59] and Chimi et al. [60].

5. Concluding remarks

A novel antioxidant regenerating system (CDH and cellobiose) was developed and demonstrated to be very effective in enhancing the quenching of the tested ROS and RNS radicals ($O_2^{\cdot-}$, OH·, ·NO). Thus, from these studies, it was demonstrated that the antioxidant regenerating system could well be used to support the regeneration of all the tested oxidized phenolic antioxidant molecules and can be exploited for continuously quenching free radicals in chronic wounds. It is strongly believed that such an antioxidant regenerating system will enable create ideal conditions for the successful healing of chronic wounds and may also provide a solution to challenges besetting cell culture studies modulating redox status of the culture environment. This can be achieved by strategically incorporating this system in wound dressing polymers like hydrogels such that when the phenolic antioxidants quench the ROS and RNS they are then regenerated by CDH or coating cell culture plates with CDH. These studies together with other ongoing studies aimed at exploiting CDH in the field of bioelectrocatalysis shows that CDH is a versatile electron transfer enzyme. Interestingly, this study also challenges the biological relevance of total antioxidant capacities of plant crude extracts measured using none biologically relevant radicals species like DPPH, ABTS, DMPD, TMAMQ, etc. when applied as medicinal remedies. This is because these total antioxidant values obtained using these methods are

greatly influenced by methoxylated phenolic antioxidant molecules like sinapic acid, ferulic acid; 2,6-dimethoxyphenol, etc. which are not able to quench $O_2^{\cdot-}$ radicals. Therefore, the values obtained if not qualified they are meaningless.

Acknowledgements

The authors are grateful for the financial support offered by EU NOVO project and the Austrian Centre of Industrial Biotechnology (ACIB).

References

[1] M. Schäfer, S. Werner, Oxidative stress in normal and impaired wound repair, Pharm. Res. 58 (2008) 165–171.
[2] E. Novo, M. Parola, Redox mechanisms in hepatic chronic wound healing and fibrogenesis, Fibrogenesis Tissue Repair 1 (2008).
[3] R. Moseley, J.R. Hilton, R.J. Waddington, K.G. Harding, P. Stephens, D.W. Thomas, Comparison of oxidative stress biomarker profiles between acute and chronic wound environments, Wound Rep. Reg. 12 (2004) 419–429.
[4] T.E. Cawston, A.J. Wilson, Understanding the role of tissue degrading enzymes and their inhibitors in development and disease, Best Pract. Res. Clin. Rheum. 20 (2006) 983–1002.
[5] B. Kumar, M. Vijayakumar, R. Govindarajan, P. Pushpangadan, Ethnopharmacological approaches to wound healing—exploring medicinal plants of India, J. Ethnopharmacol. 114 (2007) 103–113.
[6] S. Guo, L.A. DiPietro, Factors affecting wound healing, J. Dent. Res. 89 (2010) 219–229.
[7] S. Walmsley, Advances in wound management: executive summary, in: Advances in Wound Management: Executive Summary Clinica Reports, PJB Publications, Ltd., London, 2002.
[8] M.A. Fonder, G.S. Lazarus, D.A. Cowan, B. Aronson-Cook, A.R. Kohli, A.J. Mamelak, Treating the chronic wound: a practical approach to the care of nonhealing wounds and wound care dressings, J. Am. Acad. Dermatol. 58 (2008) 185–206.
[9] C. Weller, G. Sussman, Wound dressings update, J. Pharm. Pract. Res. 36 (2006) 318–324.
[10] G. Chaby, P. Senet, M. Vaneau, P. Martel, J. Guillaume, S. Meaume, L. Teot, C. Debure, A. Dompmartin, H. Bachelet, H. Carsin, V. Matz, J.L. Richard, J.M. Rochet, N. Sales-Aussias, A. Zagnoli, C. Denis, B. Guillot, O. Chosidow, Dressings for acute and chronic wounds: a systematic review, Arch. Dermatol. 143 (2007) 1297–1304.
[11] C.A. Fleck, R. Simman, Modern collagen wound dressings: function and purpose, J. Am. College Certified Wound Spec. 2 (2010) 50–54.
[12] A.D. Widgerow, Wound fluid intervention: influencing wound healing from the outside, Wound Heal. Southern Africa 4 (2011) 12–15.
[13] U. Wollina, M. Heide, W. Müller-Litz, D. Obenauf, J. Ash, Functional textiles in prevention of chronic wounds, wound healing and tissue engineering, in: P. Elsner, K. Hatch, W. Wigger-Alberti (Eds.), Textiles and the Skin, Curr. Probl. Dermatol, Karger, Basel, 2003, pp. 82–97.
[14] M. Bradley, N. Cullum, E.A. Nelson, M. Petticrew, T. Sheldon, D. Torgerson, Systematic reviews of wound care management: (2) Dressings and topical agents used in the healing of chronic wounds, Health Technol. Assess. 3 (1999).
[15] M. Bradley, N. Cullum, E.A. Nelson, M. Petticrew, T. Sheldon, D. Torgerson, Systematic reviews of wound care management: (2) Dressings and topical agents used in the healing of chronic wounds, Health Technol. Assess. 3 (1999).
[16] J. Luo, A.F. Chen, Nitric oxide: a newly discovered function on wound healing, Acta Pharmacol. Sin. 26 (2005) 259–264.
[17] P. Pacher, J.S. Beckman, L. Liaudet, Nitric oxide and peroxynitrite in health and disease, Physiol. Rev. 87 (2007) 315–424.
[18] E. Nugroho Prasetyo, T. Kudanga, W. Steiner, M. Murkovic, G. Nyanhongo, G. Guebitz, Antioxidant activity assay based on laccase-generated radicals, Anal. Biol. Chem. 393 (2009) 679–687.
[19] E. Nugroho Prasetyo, T. Kudanga, W. Steiner, M. Murkovic, W. Wonisch, G.S. Nyanhongo, G.M. Guebitz, Cellular and plasma antioxidant activity assay using tetramethoxy azobismethylene quinone, Free Radic. Biol. Med. 49 (2010) 1205–1211.
[20] E. Nugroho Prasetyo, T. Kudanga, W. Steiner, M. Murkovic, G.S. Nyanhongo, G.M. Guebitz, Laccase-generated tetramethoxy azobismethylene quinone (TMAMQ) as a tool for antioxidant activity measurement, Food Chem. 118 (2010) 437–444.
[21] E. Nugroho Prasetyo, W. Willibald, G.S. Nyanhongo, G.M. Guebitz, A unique two-way approach for the validation of total antioxidant capacity of serum samples, Eur. J. Clin. Invest. 42 (2012) 432–438.
[22] S. Amaral, L. Mira, J.M.F. Nogueira, A.P.d. Silva, M. Helena Florêncio, Plant extracts with anti-inflammatory properties—a new approach for characterization of their bioactive compounds and establishment of structure–antioxidant activity relationships, Bioorg. Med. Chem. 17 (2009) 1876–1883.
[23] D. Gazzillo, A. Giacometti, Polydisperse fluid mixtures of adhesive colloidal particles, Physica A 304 (2002) 202–210.
[24] S.J. Landis, Chronic wound infection and antimicrobial use, advances in skin & wound care, J. Prev. Heal. 21 (2008) 530–540.
[25] H. Kim, T. Kawazoe, D. Han, K. Matsumara, S. Suzuki, S. Tsutsumi, S. Hyon, Enhanced wound healing by an epigallocatechin gallate-incorporated collagen sponge in diabetic mice, Wound Rep. Reg. 16 (2008) 714–720.
[26] M.D. Phaneuf, M.J. Bide, S.L. Hannel, M.J. Platek, T.S. Monahan, M.A. Contreras, T.M. Phaneuf, F.W. LoGerfo, Development of an infection-resistant, bioactive wound dressing surface, J. Biomed. Mater. Res. A 74A (2005) 666–676.
[27] B. Halliwell, J. Gutteridge, C. Cross, Free radicals, antioxidants, and human disease: where are we now?, J Lab. Clin. Med. 119 (1992) 598–620.
[28] R. Ludwig, W. Harreither, F. Tasca, L. Gorton, Cellobiose dehydrogenase: a versatile catalyst for electrochemical applications, Chem. Phys. Chem. 11 (2010) 2674–2697.
[29] U. Temp, C. Eggert, Novel interaction between laccase and cellobiose dehydrogenase during pigment synthesis in the white rot fungus *Pycnoporus cinnabarinus*, Appl. Environ. Microbiol. 65 (1999) 389–395.
[30] F. Secundo, G. Carrea, C. Tarabiono, P. Gatti-Lafranconi, S. Brocca, M. Lotti, K.E. Jaeger, M. Puls, T. Eggert, The lid is a structural and functional determinant of lipase activity and selectivity, J. Mol. Catal. B 39 (2006) 166–170.
[31] G. Henriksson, G. Johansson, G. Pettersson, A critical review of cellobiose dehydrogenases, J. Biotechnol. 78 (2000) 93–113.
[32] L. Hilden, G. Johansson, Recent developments on cellulases and carbohydrate-binding modules with cellulose affinity, Biotechnol. Lett. 26 (2004) 1683–1693.
[33] W. Harreither, V. Coman, R. Ludwig, D. Haltrich, L. Gorton, Investigation of graphite electrodes modified with cellobiose dehydrogenase from the ascomycete *Myriococcum thermophilum*, Electroanalysis 19 (2007) 172–180.
[34] G. Safina, R. Ludwig, L. Gorton, A simple and sensitive method for lactose detection based on direct electron transfer between immobilised cellobiose dehydrogenase and screen-printed carbon electrodes, Electrochim. Acta 55 (2010) 7690–7695.
[35] C. Nistor, A. Rose, M. Farré, L. Stoica, U. Wollenberger, T. Ruzgas, D. Pfeiffer, D. Barceló, L. Gorton, J. Emnéus, In-field monitoring of cleaning efficiency in waste water treatment plants using two phenol-sensitive biosensors, Anal. Chim. Acta 456 (2002) 3–17.
[36] R. Solná, E. Dock, A. Christenson, M. Winther-Nielsen, C. Carlsson, J. Emnéus, T. Ruzgas, P. Skládal, Amperometric screen-printed biosensor arrays with co-immobilised oxidoreductases and cholinesterases, Anal. Chim. Acta 528 (2005) 9–19.
[37] M.L. Rabinovich, L.G. Vasil'chenko, K.N. Karapetyan, G.P. Shumakovich, O.P. Yershevich, R. Ludwig, D. Haltrich, Y. Hadar, Y.P. Kozlov, A.I. Yaropolov, Application of cellulose-based self-assembled tri-enzyme system in a pseudo-reagent-less biosensor for biogenic catecholamine detection, Biotechnol. J. 2 (2007) 546–558.
[38] L. Stoica, R. Ludwig, D. Haltrich, L. Gorton, Third-generation biosensor for lactose based on newly discovered cellobiose dehydrogenase, Anal. Chem. 78 (2006) 393–398.
[39] R. Ludwig, A. Salamon, J. Varga, M. Zamocky, C.K. Peterbauer, K.D. Kulbe, D. Haltrich, Characterisation of cellobiose dehydrogenases from the white-rot fungi *Trametes pubescens* and *Trametes villosa*, Appl. Microbiol. Biotechnol. 64 (2004) 213–222.
[40] Annemarie Flitsch, Endry Nugroho Prasetyo, Christoph Sygmund, Roland Ludwig, Gibson S. Nyanhongo, Georg M. Guebitz, Cellulose oxidation and bleaching processes based on recombinant *Myriococcum thermophilum* cellobiose dehydrogenase, Enzyme Microbiol. Technol. (2012).
[41] U. Baminger, B. Nidetzky, K.D. Kulbe, D. Haltrich, A simple assay for measuring cellobiose dehydrogenase activity in the presence of laccase, J. Microbiol. Methods 35 (1999) 253–259.
[42] L.H. Long, P.J. Evans, B. Halliwell, Hydrogen peroxide in human urine: implications for antioxidant defense and redox regulation, Biochem. Biophys. Res. Commun. 262 (1999) 605–609.
[43] L. Marcocci, J.J. Maguire, M.T. Droylefaix, L. Packer, The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761, Biochem. Biophys. Res. Commun. 201 (1994) 748–755.
[44] T. Nash, The colorimetric estimation of formaldehyde by means of the Hantzsch reaction, Biochem. J. 55 (1953) 416–421.
[45] G.S. Nyanhongo, E.N. Prasetyo, E. Herrero Acero, G.M. Guebitz, Reaction engineering strategies for successful development of functional polymers using oxidative enzymes, in: Reaction Engineering Strategies for Successful Development of Functional Polymers using Oxidative Enzymes, Functional Materials from Renewable Sources, ACS Books, 2012.
[46] G.S. Nyanhongo, W. Steiner, G.M. Guebitz, Biofunctionalization of polymers and their applications, Adv. Biochem. Eng./Biotechnol. 125 (2011) 97–120.
[47] Pessêgo Márcia, Ana M. Rosa da Costa, José A. Moreira, Importance of phenols structure on their activity as antinitrosating agents: a kinetic study, J. Pharm. Bioallied Sci. 3 (2011) 128–134.
[48] M.P. Rigobello, R. Stevanato, F. Momo, S. Fabris, G. Scutari, R. Boscolo, A. Folda, A. Bindoli, Evaluation of the antioxidant properties of propofol and its nitrosoderivative. Comparison with homologue substituted phenols, Free Radic. Res. 38 (2004) 315–321.
[49] S. González-Mancebo, M.P. García-Santos, J. Hernández-Benito, E. Calle, J. Casado, Nitrosation of phenolic compounds: inhibition and enhancement, J. Agric. Food Chem. 47 (1999) 2235–2240.
[50] J.S. Wright, D.J. Carpenter, D.J. McKay, K.U. Ingold, Theoretical calculation of substituent effects on the OH bond strength of phenolic antioxidants related to Vitamin E, J. Am. Chem. Soc. 119 (1997) 4245–4252.

[51] C.G.M. Heijnen, G.R.M.M. Haenen, F.A.A. van Acker, W.J.F. van der Vijgh, A. Bast, Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups, Toxicol. In Vitro 15 (2001) 3–6.
[52] Y. Sueishi, M. Hori, M. Kita, Y. Kotake, Nitric oxide (NO) scavenging capacity of natural antioxidants, Food Chem. 129 (2011) 866–870.
[53] P. Ander, G. Sena-Martins, J.C. Duarte, Influence of cellobiose oxidase on peroxidases from *Phanerochaete chrysosporium*, Biochem. J. 293 (1993) 431–435.
[54] H.Y. Aboul-Enein, I. Kruk, A. Kładna, K. Lichszteld, T. Michalska, Scavenging effects of phenolic compounds on reactive oxygen species, Biopolymers 86 (2007) 222–230.
[55] P.K.J.P.D. Wanasundara, F. Shahidi, Antioxidants: science, technology, and applications, in: Antioxidants: Science, Technology, and Applications Bailey's Industrial Oil and Fat Products, John Wiley & Sons, Inc., 2005.
[56] J. Perez-Jimenez, F. Saura-Calixto, Effect of solvent and certain food constituents on different antioxidant capacity assays, Food Res. Int. 39 (2006) 791–800.
[57] T. Ohtake, H. Etoh, Biological antioxidation mechanisms-quenching of peroxynitrite, Foods Food Ingredients J. Jpn. 208 (2003).
[58] Z. Cheng, J. Ren, Y. Li, W. Chang, Z. Chen, Study on the multiple mechanisms underlying the reaction between hydroxyl radical and phenolic compounds by qualitative structure and activity relationship, Bioorg. Med. Chem. 10 (2002) 4067–4073.
[59] V. Thavasi, R.P.A. Bettens, L.P. Leong, Temperature and solvent effects on radical scavenging ability of phenols, J. Phys. Chem. A 113 (2009) 3068–3077.
[60] H. Chimi, J. Cillard, P. Cillard, M. Rahmani, Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants, J. Am. Oil Chem. Soc. 68 (1991) 307–312.